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Expression Cloning of a Receptor for Murine Granulocyte Colony-Stimulating Factor

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Summary

Two cDNAs encoding the receptor for murine granulocyte colony-stimulating factor (G-CSF) were isolated from a CDM8 expression library of mouse myeloid leukemia NFS-60 cells, and their nucleotide sequences were determined. Murine G-CSF receptor expressed in COS cells could bind G-CSF with an affinity and specificity similar to that of the native receptor expressed by mouse NFS-60 cells. The amino acid sequence encoded by the cDNAs has demonstrated that murine G-CSF receptor is an 812 amino acid polypeptide (M_r, 90,814) with a single transmembrane domain. The extracellular domain consists of 601 amino acids with a region of 220 amino acids that shows a remarkable similarity to rat prolactin receptor. The cytoplasmic domain of the G-CSF receptor shows a significant similarity with parts of the cytoplasmic domain of murine interleukin-4 receptor. A 3.7 kb mRNA coding for the G-CSF receptor could be detected in mouse myeloid leukemia NFS-60 and WEHI-3B D^+ cells as well as in bone marrow cells.

Introduction

Production of hematopoietic cells is regulated by hormone-like growth and differentiation factors called colony-stimulating factors (CSFs). CSFs include granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and interleukin-3 (IL-3) (Metcalf, 1989; Nicola, 1989). G-CSF, produced mainly by macrophages, is important in regulating blood levels of neutrophils and in activating mature neutrophils. G-CSF stimulates some myeloid leukemia cells to proliferate or to differentiate into neutrophilic granulocytes (Nagata, 1990).

Murine G-CSF and human G-CSF have been purified (Nicola et al., 1983; Nomura et al., 1986), and cDNAs encoding G-CSF have been molecularly cloned (Nagata et al., 1986a, 1986b; Souza et al., 1986; Tsuchiya et al., 1986). Human G-CSF is a 174 amino acid polypeptide, while murine G-CSF consists of 178 amino acids. Human and mouse G-CSFs are highly homologous (72.6%) at the amino acid sequence level, in agreement with the lack of species specificity between them (Nicola et al., 1985). Although the primary structure of G-CSF does not show homology with other CSFs or growth factors, it has a weak similarity with interleukin-6 (IL-6), which stimulates proliferation and differentiation of B-lymphocytes (Nagata, 1990). Human G-CSF produced by recombinant DNA technology has proven to be a potent regulator of neutrophils in vivo using animal model systems (Tsuchiya et al., 1987; Nicola, 1989). Recent clinical trials in patients suffering from a variety of hemopoietic disorders have shown that the administration of G-CSF is beneficial in chemotherapy and bone marrow transplantation therapy (Morstyn et al., 1989).

Despite the biological importance of G-CSF, little is known about the mechanism of G-CSF-induced signal transduction in the proliferation and differentiation of neutrophilic granulocytes. Several reports in human and mouse systems have suggested that the expression of the G-CSF receptor is restricted to progenitor and mature neutrophils and various myeloid leukemia cells (Nicola and Metcalf, 1984, 1985; Nicola et al., 1985; Begley et al., 1987; Park et al., 1989). However, the G-CSF receptor has also recently been found in nonhemopoietic cells, such as human endothelial cells (Bussolino et al., 1989) and placenta (Uzumaki et al., 1989). Biochemical characterization of the G-CSF receptor has been hampered by the low number of receptors present on the cell surface (at most 1000–2000 receptors per cell). A limited number of studies have indicated that cells of the neutrophilic lineage have a single class of binding sites for G-CSF with an equilibrium dissociation constant of 100–500 pM (Nicola and Metcalf, 1984; Park et al., 1989; Uzumaki et al., 1989). Cross-linking studies of the receptor with the radiolabeled G-CSF have suggested a M_r of 150,000 for the mouse G-CSF receptor in WEHI-3B D^+ cells (Nicola and Peterson, 1986). Recently, we were able to solubilize mouse G-CSF receptor in an active form from NFS-60 cells and succeeded in purifying the receptor as a protein with a M_r of 100,000–130,000 (R. F., E. I., and S. N., unpublished data).

In this work, we isolated cDNAs encoding the murine G-CSF receptor from mouse myeloid leukemia NFS-60 cells. When transfected into COS cells, the cDNA directed expression of a receptor that has similar properties to that of the native G-CSF receptor on NFS-60 cells. The amino acid sequence of the G-CSF receptor indicates that it belongs to the recently identified growth factor receptor family (Bazan, 1989).

Results

Expression Cloning of the G-CSF Receptor cDNA

To isolate the cDNA coding for the G-CSF receptor, we used a COS cell expression system developed to isolate the murine erythropoietin receptor (D'Andrea et al., 1989). Double-stranded cDNA was synthesized using mRNA from mouse myeloid leukemia NFS-60 cells, which have relatively higher expression of the G-CSF receptor than other G-CSF-responsive myeloid leukemia cells, such as WEHI-3B D^+ or 32DC13. A cDNA library was constructed in the mammalian expression vector CDM8 (Seed, 1987) as 884 pools of 60–80 clones. Plasmid DNAs from each

pool were prepared by the boiling method and introduced into COS-7 cells grown in 6-well microtiter plates. At 72 hr posttransfection, binding reactions of ^{125}I-G-CSF (1.7×10^5 cpm [200 pM] in a 0.6 ml volume) to COS cells were carried out at 37°C for 2 hr instead of 4°C in order to obtain a greater signal (D'Andrea et al., 1989). Under these conditions, the background binding of labeled G-CSF to transfected or untransfected COS cells was routinely 308 ± 38 (SD) cpm. Plasmid DNAs from two pools (I62 and J17) yielded binding of 500 cpm and 912 cpm of ^{125}I-G-CSF, respectively, when transfected into COS-7 cells. The bacterial clones of pools I62 and J17 were arranged in 12 subgroups of 12 clones each and assayed as above. Some subgroups gave positive responses, that is, binding of 3710–4010 cpm of ^{125}I-G-CSF to COS cells. By assaying single clones from each positive subgroup, two independent clones (pI62 and pJ17) were identified. When plasmid DNAs from pI62 and pJ17 were transfected into COS-7 cells, the binding assay gave values of 30,300 cpm and 31,600 cpm, respectively.

Binding Characteristics of the Cloned Receptor

The binding characteristics of the G-CSF receptor expressed on COS cells were examined. COS cells transfected with the plasmid CDM8 or pJ17 were incubated at 4°C for 4 hr with various concentrations of ^{125}I-G-CSF in the presence or absence of at least a 500-fold excess of unlabeled G-CSF (800 nM). Untransfected COS cells or COS cells transfected with the CDM8 vector alone did not show any significant specific binding of ^{125}I-G-CSF. On the other hand, labeled G-CSF was bound at 4°C to the COS cells transfected with the plasmid pJ17. As shown in Figure 1, a Scatchard analysis of the specific binding of ^{125}I-G-CSF to COS cells revealed a single species of binding site with an equilibrium dissociation constant of 290 pM and 3.0×10^4 receptors per cell. If the transfection efficiency of COS cells was assumed to be 10%–20% (Somozapayrac and Danna, 1981), the positively transfected COS cells probably expressed the recombinant G-CSF receptor at $1.5–3.0 \times 10^5$ molecules per cell. Since the native G-CSF receptor on NFS-60 cells has an equilibrium dissociation constant of 180 pM (Figure 1D), these results suggest that the polypeptide coded by the cDNA in the plasmid pJ17 is sufficient to express the high-affinity receptor for murine G-CSF.

Human G-CSF competes with mouse G-CSF for binding to mouse WEHI-3B D$^+$ cells (Nicola et al., 1985). Accordingly, an excess of unlabeled recombinant human G-CSFs produced either by mammalian cells or Escherichia coli could compete well with labeled mouse G-CSF for binding to COS cells transfected with the plasmid pJ17 (Figure 2). No inhibition of binding of ^{125}I-G-CSF to COS-7 cells was observed in the presence of unlabeled recombinant murine GM-CSF, murine IL-3, murine IL-6, murine leukemia inhibitory factor (LIF), rat prolactin, or human M-CSF. These results correlated well with the specificity of the native G-CSF receptor or the purified receptor on NFS-60 cells (R. F., E. I., and S. N., unpublished data).

Previously, we observed that the G-CSF receptor purified from NFS-60 cells has a M_r of 100,000–130,000. To

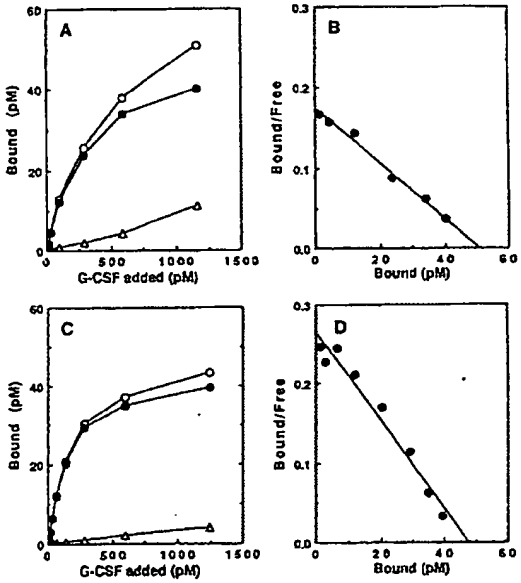


Figure 1. Binding of Radioiodinated G-CSF to COS Cells Expressing the Recombinant G-CSF Receptor and NFS-60 Cells

(A) Saturation binding of ^{125}I-G-CSF to COS cells. COS cells (1×10^6) transfected with the plasmid pJ17 were incubated with various amounts of ^{125}I-G-CSF with or without an excess of unlabeled G-CSF as described in the Experimental Procedures. The specific binding (●) is shown as the difference between total (○) and nonspecific (△) binding.

(B) Scatchard plot of G-CSF binding data in COS cells.

(C) Saturation binding of ^{125}I-G-CSF to NFS-60 cells. Total (○), nonspecific (△), and specific (●) binding to cells are shown.

(D) Scatchard plot of G-CSF binding data in NFS-60 cells.

determine the molecular size of the recombinant G-CSF receptor expressed in COS cells, chemical cross-linking of the receptor with ^{125}I-G-CSF was carried out. As shown in Figure 3, cross-linking of the G-CSF receptor on NFS-60 cells with labeled mouse G-CSF (M_r, 25,000) yielded a band with an apparent M_r of 125,000–155,000 (lane 6), indicating that the M_r of the murine G-CSF receptor on NFS-60 cells is 100,000–130,000. Similarly, cross-linking of ^{125}I-mouse G-CSF to the receptor expressed in COS cells gave a major band of M_r 120,000–150,000 (lane 4), which is slightly smaller than that detected in NFS-60 cells. These bands were not observed when the cross-linking experiment was carried out in the presence of 1.5 μM unlabeled G-CSF (lanes 2 and 5) or when the cross-linking agents were omitted (lane 3). The slightly different M_r observed in COS cells and NFS-60 cells may be explained by the differential glycosylation in these cell lines.

The Structure of Murine G-CSF Receptor

Digestions of the plasmid pJ17 and pI62 with XhoI released cDNA inserts of 3.2 kb and 3.0 kb, respectively. As shown in Figure 4A, the restriction maps of these inserts were identical except that the 5′ terminus of clone pI62 is 81 bp longer than that of pJ17 and the 3′ terminus of the clone pJ17 is 238 bp longer than that of pI62. When

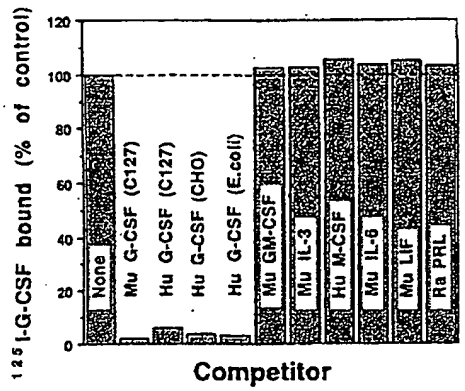


Figure 2. Specificity of G-CSF Binding to Recombinant G-CSF Receptor Expressed in COS Cells

COS cells transfected with the cDNA for the G-CSF receptor (pJ17) were incubated with 2 ng of ^{125}I-mouse G-CSF in the absence or presence of 1 μg of unlabeled murine G-CSF, human G-CSF, murine GM-CSF, human M-CSF, murine IL-6, murine LIF, or rat prolactin. As human G-CSF, human recombinant G-CSFs produced in mouse C127 cells, in Chinese hamster ovary cells, or in E. coli were used. The radioactivities bound to COS cells in each experiment are expressed as a percentage of that obtained without competitor.

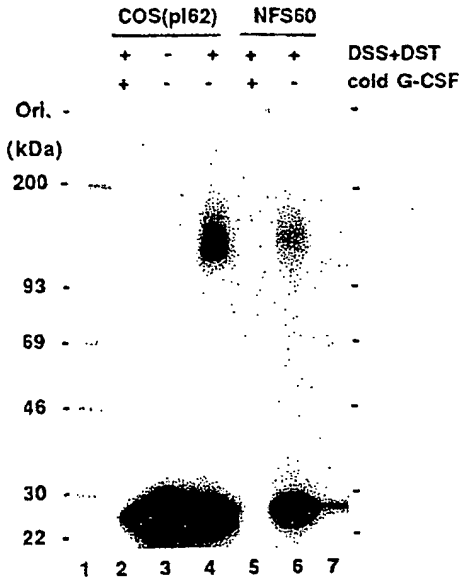


Figure 3. Chemical Cross-Linking of the G-CSF Receptor Expressed in COS and NFS-60 Cells

COS cells (5.2 × 10^5 cells/lane) transfected with the plasmid pI62 were incubated with ^{125}I-G-CSF with (lane 2) or without (lanes 3 and 4) an excess of unlabeled murine G-CSF and chemically cross-linked as described in the Experimental Procedures. Mouse NFS-60 cells (3 × 10^6 cells/lane) were similarly incubated with ^{125}I-G-CSF with (lane 5) or without (lane 6) an excess of unlabeled G-CSF and cross-linked with DSS and DST. The cell lysate was analyzed by SDS–PAGE on a 4%–20% gradient polyacrylamide gel and exposed to X-ray film at −80°C for 2 days with intensifying screens. As size markers, ^{14}C-labeled molecular weight standards (rainbow marker, Amersham) were electrophoresed in parallel (lanes 1 and 7), and sizes of standard proteins are shown in kd.

the cDNAs were sequenced, the two sequences were found to be identical within the overlapping region. Although the two cDNAs contained the complete coding sequence for the G-CSF receptor, they contained neither the poly(A) tract nor the poly(A) addition signal. The cDNA library was, therefore, rescreened by colony hybridization using the 2.5 kb HindIII–XbaI fragment of pJ17 as a probe. Fifteen positive clones were obtained from about 60,000 clones, and one of them (pF1) had 603 bp of 3′ noncoding region and contained two overlapping poly(A) addition signals. The composite nucleotide sequence of the three cloned cDNAs (pI62, pJ17, and pF1) is presented in Figure 5 together with the predicted amino acid sequence. There is a long open reading frame starting from the initiation codon ATG at nucleotide positions 180–182 and ending at the termination codon TAG at positions 2691–2693. The open reading frame (2511 nucleotides) can code for a protein consisting of 837 amino acids, including the NH_2-terminal methionine. In the 5′ sequence upstream of the long open reading frame, three other potential initiation codon ATGs can be found at positions 73, 105, and 126. All of these are followed by short open reading frames. Deletion of these ATG codons from the cDNA by digesting the plasmid pI62 with HindIII did not increase or decrease the expression level of the recombinant G-CSF receptor in COS cells (R. F. and S. N., unpublished data).

The long open reading frame starts with a stretch of hydrophobic amino acids that seems to serve as a signal se-

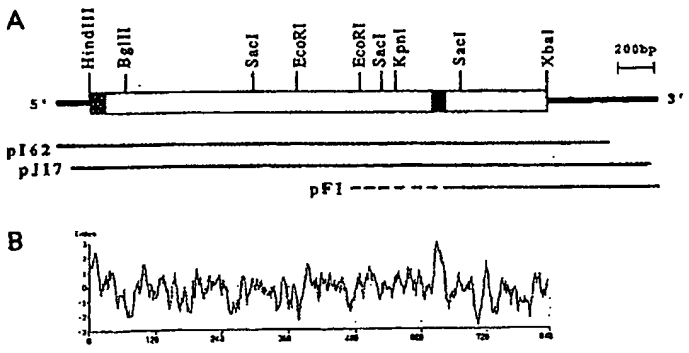
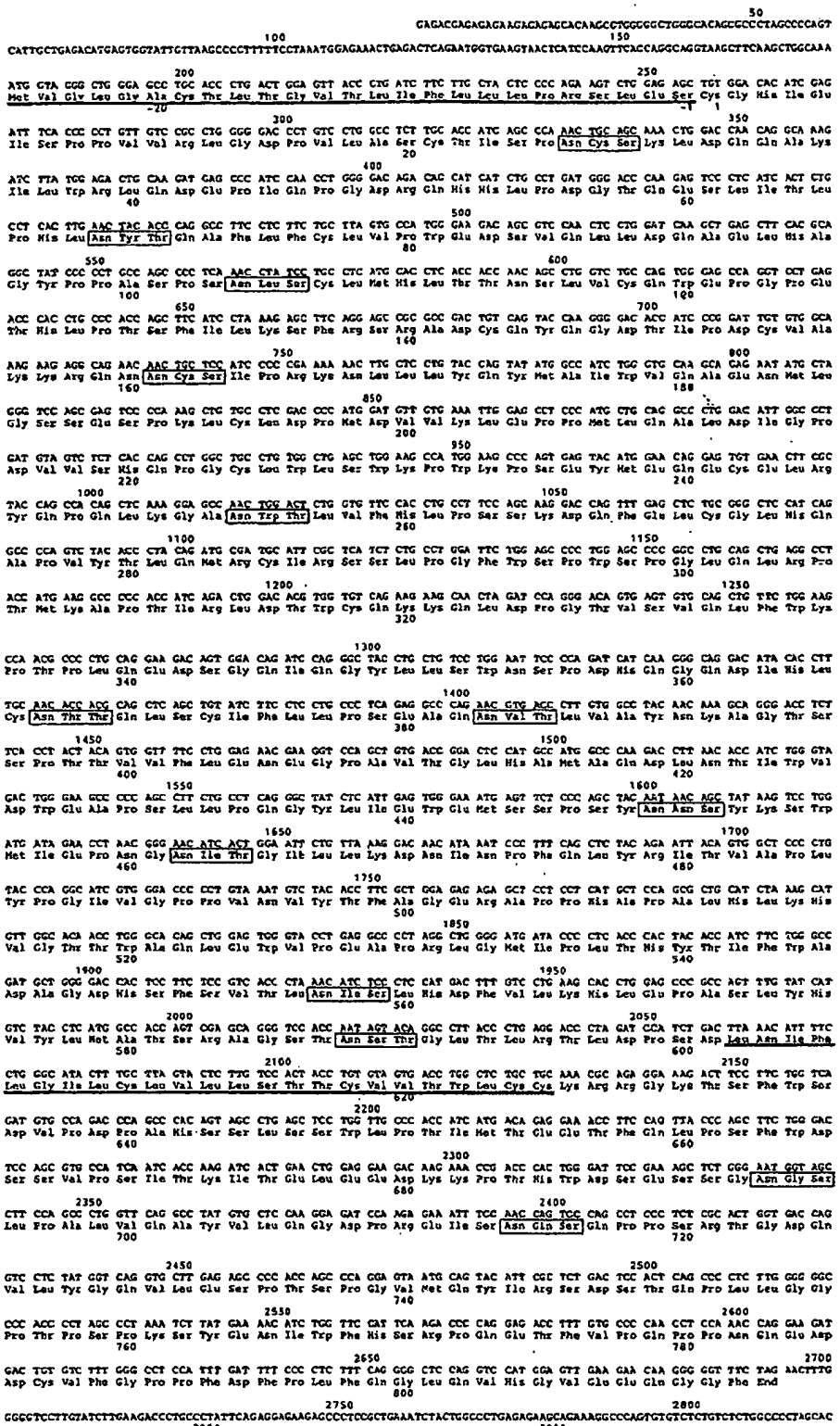


Figure 4. Murine G-CSF Receptor cDNAs

(A) Schematic representation and restriction map of three independent cDNAs (pI62, pJ17, and pF1) for murine G-CSF receptor. The box represents the open reading frame. The stippled and black regions indicate the signal sequence and the transmembrane region, respectively. The cleavage sites for restriction enzymes are shown.

(B) Hydropathy plot of the amino acid sequence of murine G-CSF receptor. The hydropathy plot was obtained by the method of Kyte and Doolittle (1982) using a window of ten residues. The numbers under the plot indicate positions of the amino acid residues of the precursor protein.



quence. By comparing the 5′ portion of the sequence with typical signal peptide cleavage sites (von Heijne, 1986), the 26th amino acid (Cys) from the initiation codon was tentatively assigned as the first amino acid of the mature protein. The mature murine G-CSF receptor thus would consist of 812 amino acids with a calculated M_r of 90,814, which is 5,000–35,000 smaller than the M_r (95,000–125,000) estimated from the cross-linking experiment (Figure 3) or the M_r of the purified murine G-CSF receptor (R. F., E. I., and S. N., unpublished data). The difference is probably due to the attachment of sugar moieties to some of the 11 putative N-glycosylation sites (Asn-X-Thr/Ser) found on the extracellular domain of the G-CSF receptor (Figure 5). A hydropathy plot (Kyte and Doolittle, 1982) of the amino acid sequence of the mature G-CSF receptor (Figure 4B) revealed a stretch of 24 uncharged amino acids extending from Leu-602 to Cys-625, which is followed by three basic amino acids. These properties are consistent with those observed in the membrane-spanning segments of many proteins (Sabatini et al., 1982). The mature G-CSF receptor thus appears to consist of an extracellular domain of 601 amino acids, a membrane-spanning domain of 24 amino acids, and a cytoplasmic domain of 187 amino acids. The NH_2-terminal half of the extracellular domain is abundant in cysteine residues (17 residues in 373 amino acids), which seems to be a feature common to the ligand-binding domain of many receptors (McDonald et al., 1989). As found in the erythropoietin receptor (D'Andrea et al., 1989), the G-CSF receptor is rich in proline (80 residues, 9.9%). Furthermore, the content of tryptophan residues in murine G-CSF receptor is relatively high (26 residues, 3.2%), although they show no particular area of localization within the receptor.

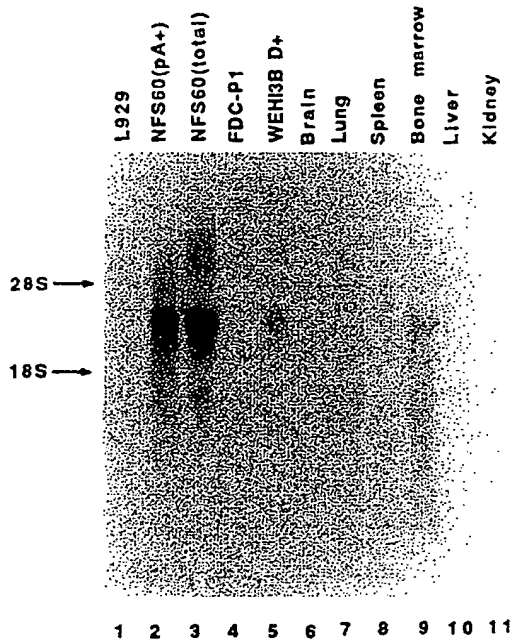


Figure 6. Northern Hybridization Analysis of Murine G-CSF Receptor mRNA

Total RNA or poly(A) RNA was prepared from mouse cell lines: L929 (lane 1), NFS-60 (lanes 2 and 3), FDC-P1 (lane 4), and WEHI-3B D$^+$ (lane 5), or from mouse tissues: brain (lane 6), lung (lane 7), spleen (lane 8), bone marrow (lane 9), liver (lane 10), and kidney (lane 11). Total RNA (30 μg) (lanes 1 and 3–11) or 2 μg of poly(A) RNA (lane 2) was electrophoresed on a 1.3% agarose gel containing 6.6% formaldehyde and analyzed by Northern hybridization as described in the Experimental Procedures.

Expression of the G-CSF Receptor mRNA

G-CSF stimulates the proliferation of mouse myeloid leukemia NFS-60 cells, while WEHI-3B D$^+$ cells can be induced to differentiate into monocytes and granulocytes by G-CSF (Nagata, 1990). To determine whether the same mRNA is expressed in NFS-60 and WEHI-3B D$^+$ cells, Northern hybridization was carried out using the cDNA from plasmid pJ17. As shown in Figure 6, a 3.7 kb mRNA could be detected in RNAs from NFS-60 cells (lanes 2 and 3) as well as from WEHI-3B D$^+$ cells (lane 5). The amount of mRNA for the G-CSF receptor is about ten times higher in NFS-60 cells than in WEHI-3B D$^+$ cells, which agrees with our observation that NFS-60 cells bind three to four times more ^{125}I-G-CSF than WEHI-3B D$^+$ cells (unpublished data). In contrast, no transcript for the G-CSF receptor was detected in RNAs from other mouse myeloid leukemia FDC-P1 cells (lane 4), which do not respond to G-CSF, or from nonhemopoietic cell lines such as L929 (lane 1) or C127I (data not shown). When mRNA expression was examined in various mouse tissues, only bone marrow cells gave a signal corresponding to the 3.7 kb mRNA (lane 9). The similar size of the mRNAs observed in bone marrow cells and NFS-60 cells suggests that the authentic mRNA for the G-CSF receptor is expressed in mouse myeloid leukemia NFS-60 cells.

Discussion

The mechanisms of signal transduction induced by various hemopoietic growth and differentiation factors, including G-CSF, are not fully understood, in part because their receptors are expressed in low levels on the cell surface. Recently, receptors for several cytokines and lymphokines have been molecularly cloned by various techniques (Yamasaki et al., 1988; Mosley et al., 1989; Hatakeyama et al., 1989; D'Andrea et al., 1989; Gearing et al., 1989; Itoh et al., 1990). To isolate the cDNA for the G-CSF receptor, we used the expression cloning method developed by D'Andrea et al. (1989). We chose this method since a single

Figure 5. Nucleotide Sequence and Predicted Amino Acid Sequence of the Murine G-CSF Receptor cDNA

Numbers above and below each line refer to the nucleotide position and amino acid position, respectively. Amino acids are numbered starting at Cys-1 of the mature G-CSF receptor. On the amino acid sequence, the signal sequence and the transmembrane domain are underlined. Two overlapping poly(A) addition signals (AATAAA) are also underlined. Potential N-glycosylation sites (Asn-X-Ser/Thr) (11 in the extracellular domain and 2 in the cytoplasmic domain) are boxed.

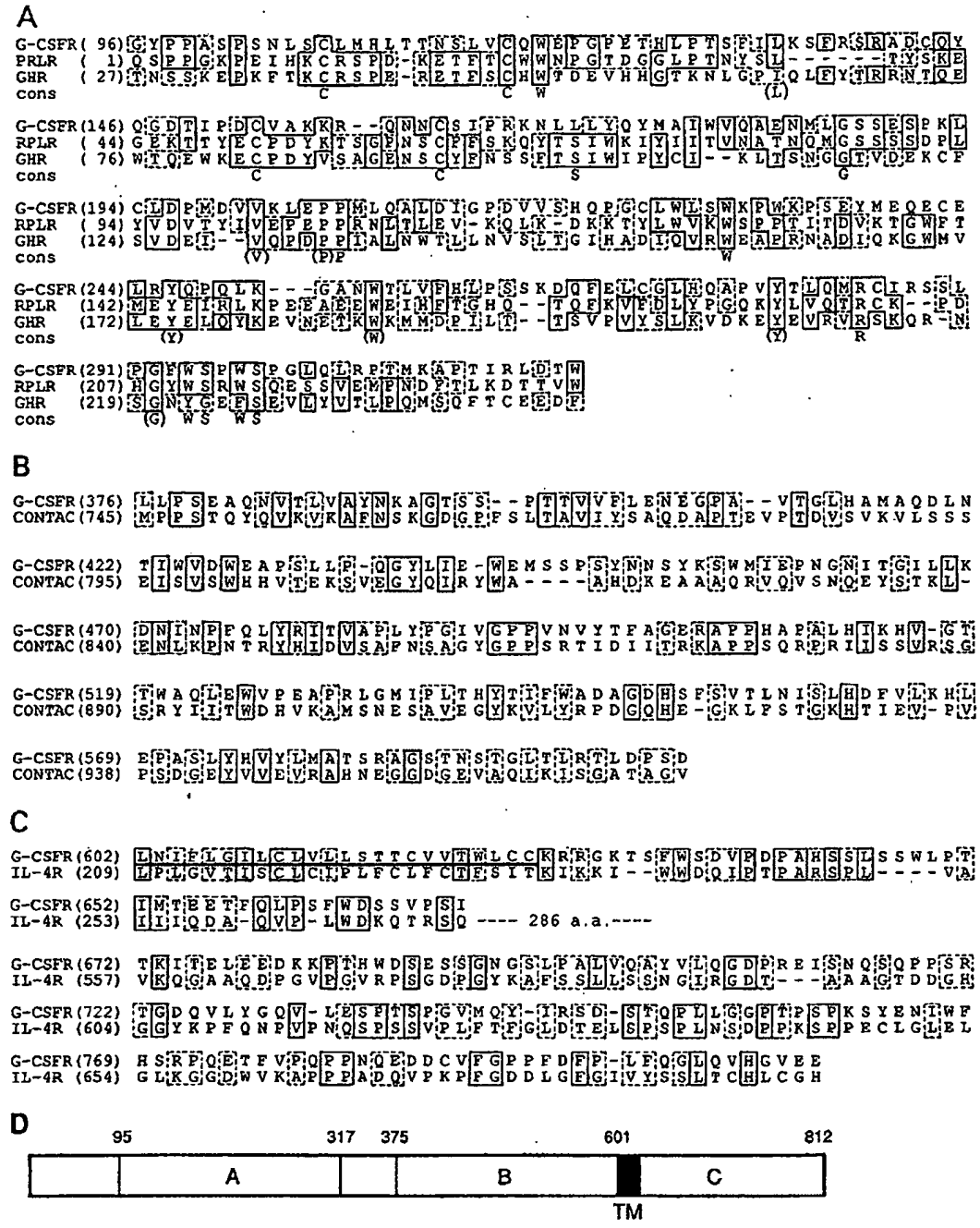


Figure 7. Comparison of the Amino Acid Sequence of the G-CSF Receptor with That of Other Growth Factor Receptors

(A) Alignment of the G-CSF receptor with prolactin and growth hormone receptors. The amino acid sequence from 96 to 317 of the murine G-CSF receptor is aligned with rat prolactin and human growth hormone receptors to give maximum homology by introducing several gaps (–). Identical residues in two sequences are enclosed by solid lines, and residues regarded as favored substitutions are enclosed by broken lines. Favored amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; F, Y, and W. Amino acids conserved in nine members of the growth factor receptor family (G-CSF, prolactin, growth hormone, erythropoietin, GM-CSF, IL-2β, IL-3, IL-4, and Il-6) are shown under each line with or without brackets. The residues without brackets are conserved in more than eight members, and the residues with brackets are conserved in five to seven members in the family.

(B) Alignment of the G-CSF receptor with contactin. The amino acid sequence from 376 to 601 of the mouse G-CSF receptor is aligned with the amino acid sequence of chicken contactin as described in (A).

polypeptide purified from NFS-60 cells is sufficient to bind G-CSF with a high affinity (R. F., E. I., and S. N., unpublished data) and little background binding of G-CSF to COS cells is observed.

Expression of the cloned cDNA gave rise to a protein that shows the same binding properties as those of the G-CSF receptor in mouse NFS-60 cells. Recombinant and native G-CSF receptors could bind ^{125}I-G-CSF with a dissociation constant of 200–300 pM (Figure 1), could specifically bind mouse G-CSF or human G-CSF, but could not bind other growth and differentiation factors such as IL-3, GM-CSF, M-CSF, IL-6, LIF, and prolactin (Figure 2). Furthermore, the cross-linking experiment indicated that the M_r of the recombinant G-CSF receptor (100,000–130,000) expressed in COS cells is similar to that of the G-CSF receptor purified from NFS-60 cells or the polypeptide cross-linked in NFS-60 cells (Figure 3). These results indicate that the G-CSF binding protein in NFS-60 cells is coded by the cloned cDNA. However, upon stimulation by G-CSF, it is not known whether this polypeptide alone can transduce the signal into cells or whether other proteins are required to form a functional receptor capable of conferring G-CSF responsiveness.

The amino acid sequence of murine G-CSF receptor (Figure 5) has many properties commonly found in the receptors for growth and differentiation factors (McDonald et al., 1989). It has a signal sequence at the N-terminal and a single transmembrane domain, suggesting that the N-terminal part of the molecule is extracellular and the C-terminal part remains inside the cell. Within the first 80 amino acids of the mature G-CSF receptor are four cysteines, two of which (Cys-21 and Cys-77) are organized in a manner characteristic of receptors of the immunoglobulin superfamily (Williams and Barclay, 1988). However, the sequence between these two cysteine residues does not seem to fulfill the criteria for recognition as a member of the immunoglobulin superfamily (Williams and Barclay, 1988).

Comparison of the amino acid sequence of the G-CSF receptor with all sequences in the National Biomedical Research Foundation data base revealed that one part of the extracellular domain of the G-CSF receptor has remarkable similarities with the prolactin receptor and another part has remarkable similarities with contactin (Figure 7). Prolactin is an anterior pituitary hormone and belongs to the family consisting of growth hormone, prolactin, and placental lactogen (Cooke et al., 1981). The amino acid sequences of human prolactin and growth hormone show a homology of 48% when conservative changes in amino acids are included. Accordingly, as shown in Figure 7A, the entire extracellular domain of the prolactin receptor (210 amino acids; Boutin et al., 1988) has a similarity of 43.2% (60 identical amino acids and 38 homologous amino acids) with a domain of the growth hormone receptor (Leung et al., 1987). To some extent, growth hormone competes with prolactin in binding to the prolactin receptor (Boutin et al., 1988) and vice versa (Leung et al., 1987). When the amino acid sequence from 96 to 317 of the mouse G-CSF receptor was aligned with the extracellular domain of the rat prolactin receptor, 54 of 227 amino acids were identical and 40 more represented conservative substitutions, yielding an overall similarity of 41.4%. The regions homologous between G-CSF and prolactin receptors are not well conserved in the growth hormone receptor, resulting in a low similarity (34.4%) between G-CSF and growth hormone receptors. Despite the similarity in amino acid sequence of the extracellular domains of the G-CSF and prolactin receptors, a 500-fold excess of unlabeled rat prolactin did not inhibit the binding of ^{125}I-G-CSF to the recombinant G-CSF receptor expressed in COS cells (Figure 2). This is consistent with the fact that the amino acid sequence of G-CSF has no significant homology to that of prolactin. These results may suggest that regions of the extracellular domain of the G-CSF receptor that are not similar to the prolactin receptor are required for the binding of G-CSF. In this regard, it is notable that the extracellular domain of the G-CSF receptor is 391 amino acids larger than that of the prolactin receptor.

When the sequences of the ligand binding domains of growth factor receptors were compiled, it was suggested that the receptors for growth hormone, prolactin, erythropoietin, and IL-6, as well as for the β chain of the IL-2 receptor, belong to a novel receptor family (Bazan, 1989). Recently isolated receptors for IL-4 (Mosley et al., 1989), IL-3 (Itoh et al., 1990), and GM-CSF (Gearing et al., 1989) are also members of this receptor family. The consensus amino acids in the family are indicated in Figure 7A. In the G-CSF receptor, the consensus cysteine and tryptophan residues are conserved, and the "WSXWS" motif (Gearing et al., 1989; Itoh et al., 1990) is also found at amino acid residues 294–298; this suggests that the G-CSF receptor belongs to the family. In this comparison of the G-CSF receptor with other hemopoietic growth factor receptors, it may be noteworthy that the similarity of the G-CSF and IL-6 receptors is less pronounced than that of the G-CSF and prolactin receptors, although G-CSF and IL-6 have a similarity of 44.6% (Nagata, 1990).

As shown in Figure 7B, the amino acid sequence from 376 to 601 in the extracellular domain of the G-CSF receptor has a significant similarity (42.9%) with a part of the extracellular domain of chicken contactin (Ranscht, 1988). Contactin is a neuronal cell surface glycoprotein of 130 kd and seems to be involved in cellular communication in the nervous system. Because the region from amino acid

(C) Alignment of the G-CSF receptor with the IL-4 receptor. The amino acid sequence from 602 to 808 of the mouse G-CSF receptor is aligned with two corresponding regions of mouse IL-4 receptor as above.
(D) Schematic representation of the mouse G-CSF receptor. The box indicates the mature G-CSF receptor. "TM" represents the transmembrane domain. Region "A" indicates a domain (222 amino acids) with similarity to other growth factor receptors, including prolactin and growth hormone receptors, and contains the "WSXWS" motif. Region "B" (226 amino acids) of the mouse G-CSF receptor shows similarity to chicken contactin. Region "C" (211 amino acids) includes the transmembrane domain (underlined) and the cytoplasmic domain of the G-CSF receptor and is similar to two regions of the mouse IL-4 receptor.

residues 737–818 of contactin can be aligned with the fibronectin type III segment involving binding to cells, heparin, and DNA, it is possible that this region plays an important role in cell adhesion (Ranscht, 1988). Granulopoiesis occurs daily in bone marrow, and the direct interaction of the neutrophilic progenitor cells with the bone marrow stroma cells has been proposed (Roberts et al., 1988). The similarity of part of the extracellular domain of the G-CSF receptor with contactin may suggest that this region is involved in the communication of neutrophilic progenitor cells and stroma cells.

The cytoplasmic domain consists of 187 amino acids and does not show any homology with the catalytic domain of the protein kinase family (Hanks et al., 1988). As observed in other growth factor receptors (Hatakeyama et al., 1989; Mosley et al., 1989), this region is rich in serine (12.8%) and proline (12.3%). When the transmembrane and cytoplasmic domains of the G-CSF receptor were aligned with the amino acid sequences of other growth factor receptors, a significant similarity with the IL-4 receptor was found. As shown in Figure 7C, the transmembrane domain and the first 46 amino acids of the cytoplasmic domain of the G-CSF receptor are homologous (50.0%) to the corresponding regions of the murine IL-4 receptor. Furthermore, amino acid residues 672–808 of the G-CSF receptor show significant similarity (45.4%) with amino acid residues 557–694 of the IL-4 receptor. These results suggest that signal transduction by G-CSF and IL-4 may be mediated by a similar mechanism.

The 3.7 kb mRNA for the G-CSF receptor was detected not only in NFS-60 cells but also in WEHI-3B D^+ cells (Figure 6), suggesting that the same G-CSF receptor is involved in G-CSF-induced proliferation of NFS-60 cells and differentiation of WEHI-3B D^+ cells. The different effects of G-CSF on NFS-60 and WEHI-3B D^+ cells may therefore be mediated by different signal transduction mechanisms downstream of the receptor. In this regard, it is interesting that the *c-myb* and *evi-1* loci, which appear to be involved in differentiation of myeloid cells, are rearranged in NFS-60 cells but not in WEHI-3B D^+ cells (Morishita et al., 1988). When RNAs from several mouse tissues were examined, the transcript for the G-CSF receptor was detected only in bone marrow cells that contain the progenitor for neutrophilic granulocytes. However, since G-CSF has some effect on bone remodeling (M. Y. Lee, R. F., T. J. Lee, J. L. Lottsfeldt, and S. N., submitted) and growth of endothelial cells (Bussolino et al., 1989), a low-level expression of the G-CSF receptor in other tissues cannot be ruled out. Under low-stringency hybridization, mRNA for the human G-CSF receptor could be detected in some human myeloid leukemia cells (R. F., Y. S., and S. N., unpublished data) using mouse G-CSF receptor cDNA as a probe. Availability of cDNA for the human G-CSF receptor would be valuable in the screening of various leukemia cells from human patients for the expression of the G-CSF receptor before treatment of the patients with G-CSF (Morstyn et al., 1989). Furthermore, the soluble form of the G-CSF receptor may be useful clinically to inhibit the proliferation of some human myeloid leukemia cells that are dependent upon G-CSF (Santoli et al., 1987).

Experimental Procedures

Cells

Mouse myeloid leukemia NFS-60 cells (Weinstein et al., 1986; kindly provided by J. Ihle, St. Jude Children's Research Hospital) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10–20 U/ml of recombinant mouse IL-3. COS-7 cells were routinely maintained in a Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

Recombinant Colony-Stimulating Factors

Human recombinant G-CSF was purified from medium conditioned with mouse C1271 cells, which were transformed with the bovine papillomavirus expression vector (Fukunaga et al., 1984) carrying human G-CSF cDNA (Tsuchiya et al., 1987). Mouse G-CSF was produced by using a similar expression system and purified to homogeneity (R. F., E. I., and S. N., unpublished data). Human recombinant G-CSF and M-CSF produced by Chinese hamster ovary cells were provided by Chugai Pharmaceutical Co. Human recombinant G-CSF produced by E. coli was purchased from Amersham. Mouse recombinant IL-3 and GM-CSF were generous gifts from Dr. A. Miyajima and K. Arai (DNAX Institute). Mouse recombinant IL-6 and mouse recombinant LIF were generously provided by Dr. T. Hirano (Osaka University) and N. Nicola (Walter Eliza Hall Institute), respectively. Rat prolactin was purchased from Chemicon International, Inc.

Mouse recombinant G-CSF was radioiodinated by the IODO-GEN method (Fraker and Speck, 1978) with a slight modification (R. F., E. I., and S. N., unpublished data). Specific radioactivities ranged from 6–8 $\times$ 10^4 cpm/ng protein (1200–1600 cpm/fmol).

Construction of the CDM8 cDNA Library

Total RNA was prepared from exponentially growing NFS-60 cells by the guanidine isothiocyanate/CsCl method (Chirgwin et al., 1979), and poly(A) RNA was selected by oligo(dT)-cellulose column chromatography. Double-stranded cDNA was synthesized as described (Nagata et al., 1986a) using a kit from Amersham, except for the reverse transcriptase, which was purchased from Seikagaku Kogyo Co. Addition of BstXI nonpalindromic linkers to the blunt-end cDNA and size fractionation of cDNA on a 1% agarose gel were performed using a kit from Invitrogen. cDNA larger than 1.8 kb was recovered from the gel and ligated to BstXI-digested CDM8 vector (Seed, 1987). E. coli MC1061/p3 cells were transformed with the ligated DNA by the electroporation method as described (Dower et al., 1988).

DNA Preparation

A total of 6 $\times$ 10^4 bacterial colonies were plated on agar at a density of 60–80 colonies per well using 24-well microtiter plates, and glycerol cultures for each pool of colonies were prepared. LB broth was inoculated with aliquots from each glycerol culture, and plasmid DNAs were prepared by the boiling method (Maniatis et al., 1982) followed by phenol extraction and ethanol precipitation.

Transfection of COS-7 Cells

Monolayers of COS-7 cells were grown in 6-well microtiter plates, and transfection of plasmid DNA into COS-7 cells was carried out by a modification of the DEAE-dextran method (Sompayrac and Danna, 1981). In brief, about 50% confluent cells were washed three times with serum-free DMEM and incubated for 8 hr at 37°C with 0.6 ml of DMEM containing 50 mM Tris–HCl (pH 7.3), 0.3 mg/ml DEAE-dextran, and 1 μg of plasmid DNA. After glycerol shock with Tris–HCl-buffered saline containing 20% glycerol for 2 min at room temperature, cells were washed twice with DMEM and incubated in DMEM containing 10% FCS.

Screening of Transfectants of COS-7 Cells

At 72 hr after transfection, COS-7 cells were washed with DMEM containing 10% FCS and 20 mM HEPES (pH 7.3) (binding medium) and incubated at 37°C for 2 hr with 1.7 $\times$ 10^5 cpm (200 pM) of ^{125}I-G-CSF in 0.6 ml of the binding medium. Unbound radioiodinated G-CSF was removed, and cells were successively washed three times with phosphate-buffered saline (PBS) supplemented with 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and once with PBS. Cells were then recovered by trypsinization, and the radioactivity associated with cells was counted using an AUTO-GAMMA 5000 MINAXI γ-counter (Packard). Background

binding of ^{125}I-G-CSF to COS-7 cells transfected with the CDM8 vector was 308 ± 38 (SD) cpm. Two positive pools were identified that showed significant binding of radioiodinated G-CSF (500 and 912 cpm) to the transfected COS-7 cells. Independent clones (144) from each positive pool were grown in six 24-well microtiter plates and subjected to sib selection (Maniatis et al., 1982) using a matrix of 12 × 12 clones. After a final round of minipreparation of plasmids and transfection into COS-7 cells, a single clone was identified from each positive pool.

Binding of ^{125}I-G-CSF to COS Cells and NFS-60 Cells

COS cells grown on 15 cm plates were transfected with 20 μg of the pl62 or pJ17 plasmid as described above except that cells were treated for 3 hr with a DEAE-dextran solution containing DNA. Cells were split into 6-well microtiter plates 12 hr after the glycerol shock and grown for 60 hr in DMEM containing 10% FCS. Cells were washed with binding medium and incubated at 4°C for 4 hr with ^{125}I-G-CSF (10 pM to 1.2 nM range) in 1.0 ml of the binding medium. To determine the nonspecific binding of ^{125}I-G-CSF to cells, a large excess of unlabeled G-CSF (800 nM) was included in the assay mixture, and the radioactivity bound to the cells was subtracted from the total binding to yield the specific binding. For binding of G-CSF to NFS-60 cells, 5.2 × 10^6 cells were incubated at 4°C for 4 hr with various concentrations of ^{125}I-G-CSF in 0.3 ml of RPMI-1640 medium containing 10% FCS and 20 mM HEPES (pH 7.3).

Chemical Cross-Linking

The chemical cross-linking of ^{125}I-G-CSF to the receptor expressed in COS cells was performed according to the procedure described for NFS-60 cells (R. F., E. I., and S. N., unpublished data). In brief, 8 × 10^5 of COS cells (on 3.5 cm plate) transfected with the plasmid pl62 were incubated at 4°C for 2.5 hr with 1.2 nM of the radioiodinated G-CSF in the presence or absence of 1.5 μM unlabeled G-CSF in 0.6 ml of the binding medium. The cells were scraped from the plate using a cell lifter and washed with 1 ml of PBS three times. Cross-linking was carried out on ice for 20 min in 1 ml of PBS containing 150 μM disuccinimidyl suberate (DSS) and 150 μM disuccinimidyl tartarate (DST). The reaction was stopped by the addition of 50 μl of 1 M Tris–HCl (pH 7.4), and cells were collected by centrifugation and lysed with 15 μl of 1% Triton X-100 containing a mixture of protease inhibitors (2 mM EDTA, 2 mM (p-aminophenyl)methanesulfonyl fluoride hydrochloride, 2 mM O-phenanthroline, 0.1 mM leupeptin, 1 μg/ml pepstatin A, and 100 U/ml aprotinin). After centrifugation, the clear lysate (10 μl) was analyzed by electrophoresis on a 4%–20% gradient polyacrylamide gel in the presence of SDS (Laemmli, 1970).

Hybridization and Nucleotide Sequence Analysis

Colony hybridization and Northern hybridization were carried out as described (Maniatis et al., 1982). As a probe, the 2.5 kb HindIII–XbaI fragment of clone pJ17 was labeled with ^{32}P by the random primer labeling method (Feinberg and Vogelstein, 1983).

DNA sequencing was performed by the dideoxynucleotide chain termination method using T7-DNA polymerase (Pharmacia) and [α-^{35}S]-dATPαS (Amersham).

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